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Physiological roles of tryptophan in teleosts: Current knowledge and perspectives for future studies

Short title: Roles of tryptophan in teleosts

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Abstract

Tryptophan is an essential amino acid with a huge functional versatility, in addition to its participation in protein synthesis. Because of the complexity of its metabolism, and the functional relevance of several of its metabolites, it directly or indirectly participates in a wide array of physiological pathways. This amino acid is a precursor for the synthesis of the neurotransmitter/neuromodulator serotonin (5HT), the hormone melatonin and kynurenine and related compounds such as kynurenic acid, quinolinic acid or niacin. Because of this, it has a key role in the regulation of processes ranging from the neuroendocrine to the immune system in vertebrates. In aquaculture, extensive research has been performed to optimize the levels of tryptophan in the commercial diets for many fish species. Providing adequate levels of this amino acid is critically important for fish growth but also for fish welfare, since tryptophan has been shown to modulate fish behaviour, stress responses, and antioxidant and immune systems. Currently available data suggest a wide variation in tryptophan requirements of different species ranging 0.3-1.2 % of dietary protein level, but recent evidence also show that fish tryptophan requirements can greatly vary depending on the rearing conditions of the fish. We also review here the participation of tryptophan and related metabolites in different physiological functions that are crucial for fish welfare. The review covers the involvement of tryptophan in 5HT- and melatonin-mediated functions, along with its participation in the regulation of the immune system and its role as an antioxidant and antitoxic agent in fish.

Keywords: tryptophan metabolism, serotonin, melatonin, antioxidant response, immune mechanism

Introduction

Tryptophan is a neutral, aromatic amino acid that is essential for animals. As an essential amino acid, tryptophan should be supplied via diet (Wilson, 2002). The teleost requirements for the different amino acids is generally determined by means of growth dose-response curves. By using this method, the dietary tryptophan requirement for several teleost has been determined to range from 0.3 to -1.2 % of dietary protein

(Table 1). In the literature, however, there are some contradictory data regarding tryptophan requirement of a number of teleost fish species (Kim *et al.*, 1987; Walton *et al.*, 1986; Walton *et al.*, 1984; Johnston *et al.*, 1990; Dabrowski, 1981; Tang *et al.*, 2013; Fatma Abidi & Khan, 2010; Murthy & Varghese, 1997; Ahmed & Khan, 2005; Benakappa & Varghese, 2003), indicating that dietary tryptophan requirement may depend on some unknown factors. On the other hand, tryptophan is the least abundant amino acid in teleosts, indicating that the requirements of tryptophan for protein synthesis are low. This suggests that growth dose-response curve might not be a suitable method for the determination of tryptophan requirements in teleosts . Different observations in previous studies are in support of this (see below).

In vertebrates, following dietary uptake , tryptophan may be used for protein synthesis and/or may enter one of the three following pathways (Fig. 1): catabolism through the kynurenine-niacin pathway, serotonin (5HT) synthesis and melatonin synthesis (Le Floc'h *et al.*, 2011). The balance among these pathways depends on physiological conditions and/or external factors. While the routes from L-tryptophan to serotonin or melatonin are well established in fish and known to be well conserved among vertebrates, much less information is available regarding the fish kynurenin-niacin pathways. Some of the components of the machinery of the mammalian kynurenin pathway are known to be present in fish (see further sections of this review) and the general catabolic routes through kynurenin are assumed to be similar to those in mammals. However, unlike mammals, fish seem to be incapable of producing relevant amounts of niacin from L-tryptophan and thus kynurenine-niacin pathway is mainly directed to remove excess tryptophan, regulate the immune response and produce sexual pheromones (Cortés *et al.*, 2016; Ng *et al.*, 1997; Yambe *et al.*, 2006).

Being the precursor of 5HT and melatonin, tryptophan modulates behaviour, mood and stress responses in fish (Lepage *et al.*, 2005a; Lepage *et al.*, 2002; Winberg & Lepage, 1998; Winberg *et al.*, 2001; Azpeleta *et al.*, 2010; Herrero *et al.*, 2007; Lepage *et al.*, 2005b). Overall, the different tryptophan metabolites have a wide range of physiological roles including modulation of stress response, antioxidant system, immune response and behavioural responses (Cortés *et al.*, 2016; Höglund *et al.*, 2005; Hoseini & Hosseini,

2010; Hoseini *et al.*, 2016; Akhtar *et al.*, 2013a; Akhtar *et al.*, 2013b; Ciji *et al.*, 2013b; Ciji *et al.*, 2012; Ciji *et al.*, 2015; Tejpal *et al.*, 2009; Wolkers *et al.*, 2012; Hoseini *et al.*, 2012; Hosseini & Hoseini, 2013; Azeredo *et al.*, 2016). The knowledge regarding tryptophan effects on teleosts is currently scarce compared to that from mammals. Many studies in teleost fish focused in the modulation of the serotonergic system and the stress response following short-term tryptophan administration. Moreover, available data show several inconsistencies in results from different studies suggesting that there is a need for more in -depth studies on tryptophan effects in teleosts.

In this review, the current knowledge on the effects of tryptophan on teleost's serotonergic, melatonergic, kynurenine, antioxidant and immune systems was summarized in order to provide a general insight on the topic for future reference.

Tryptophan requirement, growth response and deficiency signs

As an essential amino acid, dietary uptake is the main source of tryptophan for teleosts. Feedstuffs have a wide range of tryptophan content. Wheat flour (2.13% of dietary protein), soybean oilcake (1.48% of dietary protein), barley meal (1.47% of dietary protein), wheat gluten (1.3% of dietary protein), cotton seed meal (1.27% of dietary protein), blood meal (1.14% of dietary protein) and fishmeal (0.93-1.16% of dietary protein) are feedstuffs with high tryptophan content; whereas, corn (0.68-0.82% of dietary protein), hydrolysed feather meal (0.66% of dietary protein), meat and/or bone meal (0.51-0.63% of dietary protein) and corn gluten (0.47% of dietary protein) have lower tryptophan content (NRC, 2011). Using standard dose-growth response plot method [purified or semi-purified diets with a range of tryptophan content; Wilson (2002)], tryptophan requirement has been determined in a variety of teleost species, ranging 0.30-1.30% of dietary protein (Table 1). Besides fish growth retardation, dietary tryptophan deficiency leads to scoliosis, lordosis, cataract, opercular shortage, caudal fin root and interference in mineral metabolism (Fagbenro & Nwanna, 1999; Murthy & Varghese, 1997; Poston & Rumsey, 1983; Walton *et al.*, 1984; Shanks *et al.*, 1962).

Whole body amino acid profile of teleosts is suggestive of their dietary requirement (Kaushik, 1998). It is strongly constant among fish with different size, and even among

different species (Kaushik, 1998; Mambrini & Kaushik, 1995; Wilson & Cowey, 1985). However, despite this relatively high similarity in the whole-body tryptophan content tryptophan among different fish studies (in certain species or among different species), reported dietary tryptophan requirements among different species show a wide variation (0.3-1.3% of dietary protein; Table 1). Moreover, there are some contradictions in the data on dietary tryptophan requirements for certain species. For instance, in *Cyprinus carpio* dietary tryptophan requirement has been determined to be 0.3 and 1.1% of dietary protein, which stands for 4-fold variation (Dabrowski, 1981; Tang *et al.*, 2013). Similarly in *Oncorhynchus mykiss*, dietary tryptophan requirement has been reported to be 0.40-0.71 % of dietary protein (Kim *et al.*, 1987; Walton *et al.*, 1986; Walton *et al.*, 1984; Johnston *et al.*, 1990). Dietary tryptophan levels of 0.9 and 1.13% of dietary protein (Fatma Abidi & Khan, 2010; Murthy & Varghese, 1997) have been recommended as required tryptophan levels for optimum growth performance in *Labeo rohita*. However, Akhtar *et al.* (2013a) and Ciji *et al.* (2015) showed that *L. rohita* fed on diets containing tryptophan levels of 3.05-5.17% of dietary protein have significantly improved weight gain compared to control fish (fed with diet containing 0.99% tryptophan). In *Cirrhinus mrigala*, dietary tryptophan levels of 0.95 and 1.2% of dietary protein were reported as optimum dietary tryptophan levels for optimum growth of the fish. Nevertheless, Tejpal *et al.* (2009) reported that this species showed elevated growth performance when fed diets supplemented with exogenous dietary tryptophan levels of 1.94 and 3.88% of dietary protein, regardless of basal dietary tryptophan content (estimated to be 1% of dietary protein). Also, when these fish are reared under stressful conditions (e.g. increased stocking density) higher exogenous tryptophan (7.75% of dietary protein) is required to assure maximum growth performance. Such inconsistencies among dietary tryptophan requirement levels may be due to the fact that tryptophan is a functional amino acid; i.e. tryptophan is required for some other vital processes beyond protein synthesis in fish (Wu, 2009). Tryptophan is necessary for the synthesis of 5HT and melatonin; it has antioxidant properties; it is necessary to counteract toxic substances; and participates in immune function (see the following sections). A study on *C. mrigala* (Tejpal *et al.*, 2009) suggests that stress may increase fish demand for dietary tryptophan. It could be partly due to increased serotonergic activity in teleosts under

stressful conditions as well as an increased requirement for antioxidant capacity (discussed below). Akhtar *et al.* (2013a) and Ciji *et al.* (2015) reported that growth performance of *L. rohita* exposed to stressful conditions and water pollution improves when fed diets containing 3.05-5.17% tryptophan (% of dietary protein) compared to that containing 0.99% tryptophan (discussed below).

Practically, tryptophan deficiency signs (scoliosis, lordosis, cataract, opercular shortage, caudal fin root and interference in mineral metabolism) are not reported in farmed fish; because these signs are not disease-specific. However, it is necessary to monitor the effects of dietary tryptophan supplementation on fish growth performance in farm, where they face a variety of stressors (crowding, environmental and pollutant) that may increase the need for dietary tryptophan (Fig. 2).

Effects of tryptophan on 5HT synthesis, behaviour and stress response

5HT is a monoamine which is simultaneously a neurotransmitter in the central nervous system and a paracrine or endocrine signal in the gut and blood (Fernstrom, 2016). 5HT pathway is relatively less significant in terms of tryptophan consumption, but 5HT synthesis is essential regarding particular organs' well-functioning. Tryptophan uptake to fish brain is somehow similar to mammals, which takes place through a stereospecific and saturable carrier, but this carrier is used for other amino acids uptake, too (Aldegunde *et al.*, 1998). Increased tryptophan intake results in blood tryptophan elevation which in turn increases tryptophan availability for uptake into the brain (Johnston *et al.*, 1990). Tryptophan is converted to 5HT by activity of tryptophan hydroxylase and aromatic L-amino acid decarboxylase in the presence of vitamin B_6 (Johnston *et al.*, 1990). Tryptophan hydroxylase is not saturated by its substrate, tryptophan; therefore, elevated tryptophan intake causes constant increase in the brain 5HT and also 5-hydroxyindole acetic acid (5HIAA), the major serotonin metabolite (Johnston *et al.*, 1990).

About 95 % of total body 5HT content is located in the gut (Le Floc'h *et al.*, 2011), particularly in the wall where the myenteric plexus is a major monoamine producer (Caamano-Tubio *et al.*, 2007). Moreover, some, but not all teleost species (Anderson &

Campbell, 1988; Yui *et al.*, 1988), possess enterochromaffin cells in the gastrointestinal mucosa which also account for this production thereby modulating surrounding cells functions (Muñoz-Pérez *et al.*, 2016). Peripheral 5HT synthesis is also complemented by gills epithelium and both the head- and trunk- portions of the kidney (Caamano-Tubio *et al.*, 2007) as well as liver and stomach (Nagai *et al.*, 1997). In mammals, platelets can internalize and store 5HT in granules by expressing the 5HT transporter (*Sert*). Upon activation, these cells release the indoleamine which then mediates several actions from vasodilation or vasoconstriction (Berger *et al.*, 2009) to neutrophil recruitment (Duerschmied *et al.*, 2013). In contrast to mammals, fish blood-brain barrier allows the passage of 5HT to the peripheral blood vessels but, to best of our knowledge, there is no report on the presence of 5HT in fish thrombocytes (Fritsche *et al.*, 1993). However, because 5HT concentration in *O. mykiss* plasma was equal to that measured in whole blood, it is assumed in fish that 5HT coming from peripheral tissues is stored in the extracellular plasma pool (Caamano-Tubio *et al.*, 2007). Fish intestinal contraction is mediated by 5HT which, similar to mammals (Fernstrom, 2016), is mainly produced in the myenteric plexus (Velarde *et al.*, 2010). Since the presence of enterochromaffin cells in teleosts is not transversal to all species, there is a serious lack of studies on the function of enterochromaffin cells-produced 5HT. Still, given the homology between the serotonergic systems of fish and mammals, it is likely that 5HT released by these cells might display the same pro-inflammatory and motility roles it does in higher vertebrates (Bertrand & Bertrand, 2010; Gershon, 2013).

In the brain, serotonergic activity plays a major role in the hypothalamus-pituitary-interrenal (HPI) axis. Serotonergic activity is usually measured by the rate of production of 5HIAA, the main 5HT metabolite, mediated by the monoamine oxidase (Caamano-Tubio *et al.*, 2007). Tryptophan administration stimulates brain 5HT synthesis; the synthetized 5HT can both stimulate and inhibit the HPI axis as previously observed in several studies with *O. mykiss* (Lepage *et al.*, 2002; Winberg & Lepage, 1998; Winberg *et al.*, 2001), modulating aspects related to appetite and aggression (Höglund *et al.*, 2005; Höglund *et al.*, 2007). In these studies, tryptophan supplementation indirectly [through adrenocorticotropic hormone (ACTH) action] stimulated or inhibited cortisol production in unstressed or stressed fish, respectively, as observed by enhanced

serotonergic activity. Though this indirect effect induces the strongest responses, 5HT is also able to directly stimulate cortisol production in interrenal cells (Lim *et al.*, 2013). Interestingly, levels of 5HIAA increased quite simultaneously or even previously to the typical rise in plasma catecholamine concentration in stressed *O. mykiss*, suggesting that 5HT may trigger not only the HPI axis but also the response of the brain-sympathetic-chromaffin axis in fish (Gesto *et al.*, 2013). This effect is similar to that observed in turkeys (Denbow *et al.*, 1993) but not human (Rasmussen *et al.*, 1983; Benedict *et al.*, 1983).

Several studies in fish have shown that tryptophan administration increases serotonergic activity characterized by increased 5HT and/or 5HIAA (Basic *et al.*, 2013b; Herrero *et al.*, 2007; Höglund *et al.*, 2007; Hseu *et al.*, 2003; Lepage *et al.*, 2002; Lepage *et al.*, 2003), however, all these studies focused on the short-term tryptophan administration. 5HT alters voluntary feed intake, macronutrient selection, and stress response, and also suppresses aggression (Rubio *et al.*, 2006; Winberg & Nilsson, 1993; Winberg *et al.*, 1997; Øverli *et al.*, 1998). Several studies demonstrated that tryptophan administration increases 5HT and serotonergic activity and generates the effects similar to those caused by direct 5HT administration. *Gadus morhua* showed increased brain 5HIAA and 5HIAA/5HT ratio and decreased aggressive behaviour when treated with tryptophan for 7 days (Höglund *et al.*, 2005). Such an aggression-suppressive effect was also observed in *Brycon cephalus* after 7 days tryptophan administration, although serotonergic activity was not reported (Wolkers *et al.*, 2012). Similarly, *Epinephelus coioides* treated with tryptophan for 10 days showed suppressed aggressive behaviour and cannibalism along with brain 5HT elevation (Hseu *et al.*, 2003). Also, *Dicentrarchus labrax* showed increase in 5HT synthesis and altered voluntary locomotion after 7 days tryptophan administration (Herrero *et al.*, 2007). These studies suggest that tryptophan affects teleost behaviour via the serotonergic system; however, it is necessary to monitor long-term tryptophan administration on the fish serotonergic response and behaviours.

Tryptophan requirement increases under stressful condition as a decrease in plasma concentrations of tryptophan was observed in fish under stressful husbandry conditions when compared to non-stressed fish (Aragao *et al.*, 2008; Costas *et al.*, 2008), thus

dietary tryptophan supplementation may be a promising nutritional strategy for welfare management in aquaculture (e.g. high rearing densities, transport and handling). Most of the studies on tryptophan effects on the fish stress response have focused on short-term tryptophan administration (7-15 days), but both acute and chronic stressors. In *O. mykiss*, 7 days tryptophan administration had no significant effects on blood cortisol, but suppressed post-acute-stress cortisol elevation, and this change in stress response seems to be modulated via serotonergic activity and ACTH release (Lepage *et al.*, 2002; Lepage *et al.*, 2003). Such an effect was observed in *D. labrax* fed a tryptophan-supplemented diet for 7 days (Herrero *et al.*, 2007). Also, in *Salmo trutta*, 7 days tryptophan administration counteracted stress-induced anorexia (Höglund *et al.*, 2007). But there are some studies showing different results. In *Oreochromis niloticus*, 7 days feeding with a tryptophan-supplemented diet increased serotonergic activity, decreased basal cortisol, but had no significant effects on post stress cortisol (Martins *et al.*, 2013). In *Salmo salar*, 7 days tryptophan administration had no significant effects on basal and post stress (acute) brain serotonin, 5HIAA and 5HIAA/5HT ratio, while it either stimulated or inhibited basal and post stress plasma cortisol, depending on tryptophan level and time (Basic *et al.*, 2013a). Using the same tryptophan levels in *G. morhua*, Basic *et al.* (2013b) found elevated 5HIAA/5HT ratio depending on tryptophan concentration. Tryptophan had no significant effects on basal cortisol, but mitigated post stress cortisol response 6 days, but not 1 and 2 days, after tryptophan administration termination; suggesting long lasting effects tryptophan on stress response. Similarly in *S. salar*, post stress cortisol levels decreased 8 and 21 days after a 7-day period tryptophan administration (Höglund *et al.*, 2017). The importance of tryptophan administration time on serotonergic activity and cortisol response has been also reported earlier in *O. mykiss* (Lepage *et al.*, 2003). Interestingly, Hoseini *et al.* (2016) reported increase in basal and post stress cortisol levels in *Acipenser persicus* after 15 days feeding with tryptophan-supplemented diet (0.5% of diet). The same authors Hoseini and Hosseini (2010), however, reported a mitigation of the stress response (serum cortisol and glucose) to a 7-days osmotic challenge in *C. carpio* fed with similar tryptophan supplementation (0.5% of diet). On the other hand, only a few studies have evaluated the effects of long-term tryptophan administration on the fish stress response. Morandini *et al.* (2015) found suppressed basal cortisol and

increased brain serotonergic activity after 4 weeks tryptophan administration in *Cichlasoma dimerus*, with no gender-specific trend. Tejpal *et al.* (2009), Akhtar *et al.* (2013a) and Kumar *et al.* (2014) showed that 45-90 days tryptophan administration under constant stressful conditions significantly suppressed cortisol and glucose responses and improved growth performance in *C. mrigala* and *L. rohita*, suggesting the need for higher tryptophan intake under stressful conditions to support maximum growth (Fig. 2).

Altogether, these studies show that there is a need for further research to find important factors affecting tryptophan effects on the fish stress response, from brain serotonergic activation to the release of cortisol. As mentioned above, timing appears to be a critical factor; and due to time-dependent nature of serotonergic and cortisol responses to stress, administration and sampling time should be taken into account when tryptophan effects on acute stresses are investigated. Also, it seems that tryptophan administration level needs to be considered as the above mentioned studies used a wide range (0.3 % - 4.45 %) of tryptophan supplementation. The matter is even more complicated when species differences emerge, as two similar studies with similar tryptophan levels but different species and exposure times showed different outputs (Basic *et al.*, 2013a; Basic *et al.*, 2013b).

Tryptophan and melatonin synthesis

Melatonin is produced from 5HT and is a rhythmic hormonal signal involved in the synchronization of daily processes in which the pineal organ and retina play a central role. Therefore, the amino acid tryptophan is also an indirect precursor for the biosynthesis of the hormone melatonin (N-acetyl-5-methoxytryptamine), which could be partly responsible for the effects observed after tryptophan administration in vertebrates. In melatonin-producing cells, tryptophan is first transformed to 5HT (Fig. 1), which is subsequently transformed into melatonin through two enzymatic steps: the transformation of serotonin into N-acetylserotonin, catalyzed by aryl-alkylamine N-acetyl transferase (AANAT; EC 2.3.1.87), and the subsequent conversion of N-acetylserotonin into melatonin, catalyzed by the hydroxyindole-*O* -methyltransferase (HIOMT; EC 2.1.1.4) (Falcón *et al.*, 2010).

Pineal melatonin content elevates at night but decreases with light. Plus, because melatonin is highly lipophilic and can therefore easily cross cell membranes, its plasma levels oscillates parallel to pineal production (Falcón *et al.*, 2011). On the other hand, melatonin produced in the gut and retina is considered to have paracrine actions in those tissues. However, gut melatonin seems somehow to have contribution in circulating melatonin level in fish. The gastrointestinal tract is a major melatonin production site. Gastrointestinal cells are probably responsible for low, daytime levels of melatonin, while pinealocytes light-sensitive melatonin production accounts for most of the night-time hormone levels.

Other external and internal factors may account for melatonin activity modulation, though instead of dictating rhythms, their action is limited to modifying rhythms amplitude (Falcón *et al.*, 2011). These can be temperature, hormones (melatonin itself) and catecholamines (Falcón *et al.*, 1991).

In teleosts, melatonin is engaged in several physiological processes other than those ruled by circadian rhythms, from gut motility (Muñoz-Pérez *et al.*, 2016) to osmoregulation (Kulczykowska *et al.*, 2006), immunity (Cuesta *et al.*, 2008; Cuesta *et al.*, 2007), stress response (López-Patiño *et al.*, 2013) and antioxidant capacity (Maitra & Hasan, 2016), feed intake (Pinillos *et al.*, 2001; Velarde *et al.*, 2010) and voluntary activity (Herrero *et al.*, 2007). Still, a large variability among responses, regulatory mechanisms and enzyme distribution is observed in different teleost species.

Tryptophan administration has been shown to increase melatonin concentration in blood and in other tissues such as the pineal gland or the gastrointestinal tract. For example, oral administration of tryptophan has been shown to increase blood and tissue levels of melatonin in birds and mammals (Paredes *et al.*, 2007a; Sanchez *et al.*, 2008; Huether *et al.*, 1992; Hajak *et al.*, 1991). However, the effect of tryptophan administration on tissue melatonin content could vary with the moment of the day or the reproductive stage (Reiter *et al.*, 1990; Guchhait & Haldar, 2001). In fish, few existing studies resulted in ambiguous conclusions. As in mammals, and similarly to what happens with 5HT, tryptophan administration leads to increased melatonin production in the *O. mykiss* enterochromaffin cells (Lepage *et al.*, 2005b; Acuna-Castroviejo *et al.*, 2014). Lepage *et al.* (2005b) observed that melatonin levels were not different at night in *O. mykiss* fed

tryptophan supplemented diets, in contrast to what happened during daytime. The authors suggested two hypothesis; either i) intestinal melatonin production was decreased during the night, or ii) melatonin itself acted in a negative feedback mechanism on pineal hormone synthesis, the second being supported by observations in both birds (Wright & Preslock, 1975) and fish (Yanez & Meissl, 1995). In contrast to *O. mykiss*, tryptophan did not alter the hormone synthesis in the gut of the *D. labrax* (Herrero *et al.*, 2007). The authors suggested that the lack of increase in melatonin levels could be related to the low level of tryptophan supplementation utilized in this study (tryptophan content in the experimental diet was increased 2-fold with respect to the control diet), compared to the previous study in fish [Lepage *et al.* (2005b); a 8-fold increase in the tryptophan-enriched diet compared to the control diet].

Melatonin administration in fish and other vertebrates has been shown to induce some stress-mitigating effects that resemble some of the effects observed after tryptophan treatment. In birds and mammals, melatonin has been demonstrated to act as a central and peripheral inhibitor of the hypothalamus-pituitary-adrenal (HPA) axis. Animals treated with melatonin usually display an attenuated glucocorticoid response to stress (Konakchieva *et al.*, 1997; Torres-Farfan *et al.*, 2003; Saito *et al.*, 2005), in a similar fashion to what is observed after tryptophan treatment [Dantzer and Moermede (1979); Markus *et al.* (2002); also refer to previous section of this review]. In teleost fish, there is also a body of evidence suggesting an anti-stress effect for melatonin at the level of the HPI axis. In this regard, melatonin has been shown to inhibit and/or delay the glucocorticoid response to stress in fish (Azpeleta *et al.*, 2010; López-Patiño *et al.*, 2013; Conde-Sieira *et al.*, 2014; Gesto *et al.*, 2016). Again, this effect is similar to what is observed after tryptophan treatment in fish (Herrero *et al.*, 2007; Lepage *et al.*, 2002; Lepage *et al.*, 2003). The mechanisms behind the observed melatonin effects on the HPA/HPI axis are not well known. Some evidences in mammals point to a direct effect of melatonin on the adrenal gland (Appa Rao *et al.*, 2001; Torres-Farfan *et al.*, 2003), but there is also evidence suggesting that melatonin could have a direct action at the brain, modulating the central pathways controlling the HPA/HPI axis (Xu *et al.*, 1996; Conde-Sieira *et al.*, 2014; Gesto *et al.*, 2016).

Based on the similitudes between the stress response-mitigating effects of tryptophan and melatonin treatment, and on the fact that tryptophan treatment increases melatonin levels in vertebrates, it seems logical to think that melatonin could be at least partly responsible for the stress-related effects of tryptophan, even when those effects are generally attributed to the modulation of the serotonergic system (see previous section of this review). However, the extent of melatonin participation in the effects induced by tryptophan administration is not well known. In an elegant series of studies, Lepage *et al.* (2005b) first suggested the possibility that melatonin could be mediating the stress- and aggression- related effects of tryptophan in *O. mykiss* but, in a subsequent study, they showed that most of the effect was probably being mediated by 5HT (Lepage *et al.*, 2005a). However, even when it seems clear that the involvement of melatonin is not required for tryptophan to exert its stress-mitigating effects, a complementary action of both serotonergic system and melatonin mediating the anti-stress action of tryptophan administration should not be ruled out. Since the increase of melatonin levels after tryptophan treatment seems to be dose-dependent (Herrero *et al.*, 2007; Lepage *et al.*, 2005a) a diet supplementation of tryptophan high enough to increase circulation/tissue melatonin levels could be acting on the stress axis by both 5HT- and melatonin-mediated mechanisms.

Melatonin could be also involved in other effects observed after tryptophan administration in fish. For example, it could be mediating part of the effects of tryptophan regarding its antioxidant properties and its effects on the immune system (see the following sections in this review).

Furthermore, the potential effects of a sustained elevation of melatonin levels after long-term tryptophan supplementation on fish growth and other physiological aspects are not known. Besides its known involvement in the circadian and seasonal regulation of different physiological processes (Falcón *et al.*, 2007), melatonin has been also shown to affect feeding and aggressive behaviour in fish (Munro, 1986; López-Olmeda *et al.*, 2006; Pinillos *et al.*, 2001). Further research would be needed to unveil whether any of those aspects could be affected by a long-term increase in circulating melatonin levels in fish.

Antioxidant capability of tryptophan

In aquaculture, fish are exposed to multiple stressors derived from farming management which can lead to reactive oxygen and nitrogen species (ROS and RNS) generation and then to an oxidative stress situation (Andrade *et al.*, 2015; Castro *et al.*, 2012; Morales *et al.*, 2004; Pérez-Jiménez *et al.*, 2012; Wu *et al.*, 2015). To counteract the possible negative effect of ROS overproduction, fish have evolved effective defence systems, consisting of both enzymatic defences such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPx; EC 1.11.1.9), glutathione reductase (GR; EC 1.8.1.7) and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), and non-enzymatic antioxidant defences including vitamins, carotenoids, glutathione, thioredoxins, among others (Halliwell & Gutteridge, 2015; Martínez-Álvarez *et al.*, 2005). When oxidant agents exceed antioxidant defences, oxidative stress takes place and macromolecules in cells are damaged (Halliwell & Gutteridge, 2015) and this fact might reduce growth performance and feed intake of animals, affect the quality and palatability of the final product, increase susceptibility to diseases or even lead to death, with the considerable economic loss for aquaculture producers (Andrade *et al.*, 2015; Castro *et al.*, 2012; Suárez *et al.*, 2009). Although different strategies to mitigate oxidative stress effects can be adopted, nutrition approach plays a central role in this struggle (Oliva-Teles, 2012).

In fish, dietary supplementation with amino acids have been shown to improve antioxidant capacity enhancing the activity of main antioxidant enzymes and glutathione and decreasing oxidative damage in lipids and proteins, in different fish species (Coutinho *et al.*, 2016; Giri *et al.*, 2015; Pérez Jiménez *et al.*, 2012). In the case of tryptophan, although there are many studies in different fish species focused on its nutritional requirements, role in behaviour, immunological properties or primary stress modulation (see the other sections of this review), very few reports are available about its effect on oxidative stress mitigation in fish (Akhtar *et al.*, 2013a; Ciji *et al.*, 2015; Jiang *et al.*, 2016a; Jiang *et al.*, 2015; Kumar *et al.*, 2014; Wen *et al.*, 2014).

It has been proposed that tryptophan exhibits antioxidant activity *per se*, since it reacts with highly reactive free radicals such as hydroxyl radicals and also modulates

antioxidant enzyme activities (Del Angel-Meza *et al.*, 2011; Peyrot & Ducrocq, 2008; Reyes-Gonzales *et al.*, 2009). This fact has been shown both *in vitro* and *in vivo* studies with terrestrial animals including mammals and birds. For example in rabbits, tryptophan was effective in protecting the animals from the generation of free radicals and lipid oxidative damage produced by a hypoxic myocardial injury (Narin *et al.*, 2010). In weaned piglets, increasing levels of dietary tryptophan ameliorated the hepatic oxidative stress situations induced by injection of diquat (Mao *et al.*, 2014). Liu *et al.* (2015) observed that dietary tryptophan supplementation improved antioxidant status of ducks. However, the mechanisms by which tryptophan exerts its antioxidant capacity are not clear and need to be elucidated.

It is known that many of the potential antioxidant properties of tryptophan have been attributed to its metabolites (Pérez-González *et al.*, 2014). In this sense, 5HT and melatonin, which are the main molecules synthetized from tryptophan, have been shown as important antioxidants, although compared to melatonin, 5HT antioxidant capacity has been less explored. Several studies, both *in vitro* and *in vivo*, have shown that 5HT can act as a direct free radical scavenger, mainly of superoxide anion, hydrogen peroxide and hydroxyl radicals and decrease lipids oxidative damage in several tissues (Daniels *et al.*, 1996; Gülçin, 2008; Park *et al.*, 2002). Moreover, 5HT has been shown to exert its antioxidant action through chelating activities of ferrous ions (Sarikaya & Gulcin, 2013). Additionally, its direct metabolite, N-acetyl-serotonin, has been also indicated as antioxidant molecule by different authors (Lezoualc'h *et al.*, 1998; Moosmann *et al.*, 1997).

Melatonin has different physiological roles in fish (see previous section in this review) and it is the most important antioxidant molecule of those derived from tryptophan metabolism. Unlike other antioxidants, it can scavenge up to ten free radicals, including ROS and RNS, throughout a cascade of reactions that converts this indole in other secondary and tertiary metabolites which are also able to neutralize free radicals (Tan *et al.*, 2015). Moreover, melatonin is not only an effective oxidant agent scavenger, but also has the ability of activating main antioxidant enzymes such as SOD, CAT and GPx, fact that has been demonstrated in humans and different terrestrial animals including

mammals and birds (Barlow-Walden *et al.*, 1995; Cabeza *et al.*, 2001; Fjaerli *et al.*, 1999; Paredes *et al.*, 2007b).

In the same way, other studies about tryptophan metabolites, such as 5-hydroxytryptophan, indole-3-acetic acid, 3-hydroxyanthranilic acid, 3-hydroxykynurenine or xanthurenic acid have demonstrated their antioxidant capacities both as ROS scavengers and modulation of antioxidant enzymes (Christen *et al.*, 1990; Ferrari *et al.*, 2014; Matuszak *et al.*, 1997; Pérez-González *et al.*, 2015; Peyrot & Ducrocq, 2008). Similarly, some tryptophan-metabolizing enzymes, such as tryptophan 2,3-dioxygenase (TDO; EC 1.13.11.11) and indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.52) has been considered as antioxidant since in their reactions to catalyse the oxidative degradation of tryptophan, they use the superoxide anion and hydrogen peroxide as cofactors (Batabyal & Yeh, 2007; Britan *et al.*, 2006; Hayaishi, 1996).

The general effects of dietary tryptophan supplementation (beyond to normal requirements) in oxidative status of cultured fish have been scarcely studied (to our best knowledge only in two fish species, *L. rohita* and *Ctenopharyngodon idella*). Akhtar *et al.* (2013a) studied the effect of different levels of dietary tryptophan supplementation (0, 0.7 and 1.4%) in *L. rohita* under stressful conditions of temperature and salinity, observing that SOD and CAT activities in liver and gills were reduced in tryptophan supplemented groups, concluding that this amino acid had a role in the oxidative stress-mitigation function. A later study in the same fish species under thermal stress and fed on dietary tryptophan supplemented levels of 0, 0.36, 0.72 and 1.42 %, confirmed the previous observed results, observing SOD and CAT activities in liver and gills significantly higher in the control groups that decreased with the increase of dietary tryptophan inclusion (Kumar *et al.*, 2014). In the same way, the activities observed for SOD and CAT in muscle, liver and brain of *L. rohita* kept under nitrite exposure-induced stress, increased in fish fed on 0% dietary tryptophan supplementation group, whereas the activity of these antioxidant enzymes was decreased when fish were fed on 0.75 and 1.5% of dietary tryptophan supplemented levels (Ciji *et al.*, 2015). These studies have used tryptophan levels higher than the species requirement suggesting that

tryptophan requirement increases under oxidative conditions caused by environmental stress and pollutants (Fig. 2).

In *C. idella*, Wen *et al.* (2014) evaluated the effects of dietary tryptophan (0.07, 0.17, 0.31, 0.4, 0.52 and 0.61% inclusion levels) in the intestinal oxidative status after stress induced by environmental copper exposure. The fish fed on dietary tryptophan supplementation increased CuZn-SOD and GPx activities and GSH contents and also decreased the oxidative damage biomarkers for protein and lipids determined as protein carbonyl and malondialdehyde (MDA) levels. Moreover, the relative gene expression levels of CuZn-SOD and GPx were up-regulated with increasing of dietary tryptophan up to a certain level, concluding that appropriate dietary levels of this amino acid improves antioxidant status in fish. Additionally, in this study, the authors determined that up-regulation of intestine antioxidant enzyme gene expression might be related to increase of mRNA levels of Nrf2 observed in fish fed on dietary tryptophan levels up to 0.44%. Nrf2 is a signal molecule which has been related to expression of genes that encode antioxidant enzymes (Lushchak, 2011). In this sense, a positive correlation between CuZn-SOD and GPx and gene expression level of Nrf2 was observed (Wen *et al.*, 2014).

In later studies, Jiang *et al.* (2015) determined the effect of dietary tryptophan supplementation on antioxidant defence mechanisms, oxidative damage biomarkers and related signalling molecules, Nrf2 of gill in *C. idella*. The results showed that adequate tryptophan levels improved gill oxidative status avoiding oxidative damage, since ROS level decreased in fish fed on this amino acid compared to un-supplemented group. These authors related the obtained results with the scavenger capacity of tryptophan *per se*, neutralizing superoxide and hydroxyl radicals. Additionally, oxidative status improvement was also justified by the results observed in GSH content, CuZn-SOD, CAT, GPx, GST and GR activities, which were increased with dietary tryptophan supplementation compared to the control group, and that were positively correlated to the mRNA levels for these enzymes. As for the case of *L. rohita*, in *C. idella*, Nrf2 expression was also up-regulated by dietary tryptophan, which could partially justify the increase in enzymatic antioxidant defenses. On the other hand, whereas in muscle of *C.*

idella, responses for MDA levels, protein carbonyl content, GPx activity and expression and GSH levels were similar to those previously observed in gills, no changes in either SOD activity or gene expression were observed (Jiang *et al.*, 2016b). Even more, CAT activity and gene expression decreased at optimal dietary tryptophan levels, fact that was justified by the complementary action of GPx.

Altogether, these results might suggest that amino acid supplementation in general, and tryptophan in particular, is a very positive practice to improve animal welfare throughout the optimization of the oxidative status. However, literature also includes studies with contradictory results that point out the necessity of researching the conditions in which the amino acid supplementation is performed (Deng *et al.*, 2014; Jiang *et al.*, 2016a; Jiang *et al.*, 2015; Jiang *et al.*, 2016b; Peyrot & Ducrocq, 2008). In fish species, Jiang *et al.* (2015) and Jiang *et al.* (2016b) observed that excessive tryptophan inclusion in diets leads to oxidative stress in gills and muscle of *C. idella*. So, when compared to the optimum levels of tryptophan (0.31-0.4%), excessive supplementation of this amino acid (0.61%) generate increased ROS content, MDA and protein carbonyl levels and low GSH content and SOD, CAT, GPx, GST and GR activities and gene expression levels. In these cases, the authors indicated that a possible motive for the generation of this oxidative stress situation might be related to the production of 2,3-pyridine dicarboxylic acid, a metabolite of tryptophan that has been demonstrated as stimulator of ROS production (Sadeghnia *et al.*, 2013; Santamaría *et al.*, 2001).

Tryptophan antitoxic effects

A few studies have been conducted to find if dietary tryptophan administration counteracts adverse effects of toxic substances in teleosts. Hoseini *et al.* (2012) showed that dietary tryptophan administration significantly improved *C. carpio* survival upon copper exposure. Also, tryptophan suppressed some adverse effects of copper toxicity on serum cortisol, glucose and alanine transaminase (ALT). Fatahi and Hoseini (2013) found that tryptophan supplementation significantly improves *Rutilus caspicus* survival following acute copper exposure. Ciji *et al.* (2013b) demonstrated that dietary tryptophan supplementation prevents nitrite-induced haematocrit, total protein, albumin and globulin demotion in *L. rohita*. Ciji *et al.* (2013a) observed that dietary tryptophan

supplementation in *L. rohita* mitigates growth deterioration, cortisol elevation, T_4, testosterone and estradiol demotion under nitrite exposure. Ciji *et al.* (2015) observed that dietary tryptophan mitigates nitrite-induced growth and feed efficiency deterioration in *L. rohita*. Also the added tryptophan significantly mitigated adverse effects of nitrite exposure on blood white blood cell count; serum total protein, albumin, globulin, glucose and lysozyme; liver CAT, SOD, alanine transaminase (ALT) and aspartate transaminase (AST); and muscle lactate dehydrogenase, ALT and AST.

Although the aforementioned studies suggest that tryptophan supplementation is beneficial for fish when exposed to toxicants, the underlying mechanisms have not been studied. It is known that some of those toxicants may interfere with brain serotonergic activity and suppress 5HT synthesis as reported in *C. carpio* exposed to ambient copper (De Boeck *et al.*, 1995), *Channa punctatus* exposed to carbofuran (Gopal & Ram, 1995) and *Oreochromis mossambicus* exposed to mercury (Tsai *et al.*, 1995). Therefore, the serotonergic activity-promoting ability of tryptophan administration could help to counteract the adverse effects of those toxic substances on the fish serotonergic activity. This hypothesis is yet to be explored in fish.

Another potential explanation for the positive effects of tryptophan in fish exposed to toxic substances might be mitigation of oxidative stress through its antioxidant property, as described above. This is because the toxic agents used in the commented studies are all known to cause oxidative damage in fish (Jia *et al.*, 2015; Ciji *et al.*, 2012; Vutukuru *et al.*, 2006; Wei & Yang, 2016; Roméo *et al.*, 2000; Sanchez *et al.*, 2005; Ciji *et al.*, 2015).

Tryptophan and immune response

There has been growing interest in recent years in the role of tryptophan in both stress and immune functions. The neuroendocrine–immune network is essential for homeostasis during stress and infection and thus sustains a balanced immune response to effectively clear the pathogen, while minimizing the damage to the host (Verburg-Van Kemenade *et al.*, 2009). A number of different hormones and cytokines, as well as their interactions, are involved (Dhabhar, 2009). The energetic costs of the stress condition will activate

560 other hormones involved in metabolic support, which may also have an influence on
561 immune function. Therefore, leucocytes express glucocorticoid, adrenergic, cholinergic and
562 opioid receptors, which make them sensitive to a wide repertoire of neuroendocrine
563 responses (Verburg-Van Kemenade *et al.*, 2009). For instance, glucocorticoids are known
564 to have anti-inflammatory and immunosuppression actions in vertebrates. In this regard,
565 cortisol alone can influence multiple aspects of immune defence mechanisms in fish.
566 Cortisol inhibits lipopolysaccharides (LPS)-induced expression of pro-inflammatory
567 cytokines and inducible nitric oxide (NO) synthase (Verburg-van Kemenade *et al.*, 2011;
568 Stolte *et al.*, 2008). Cortisol also induces apoptosis and inhibits proliferation of immune
569 cells (Verburg-Van Kemenade *et al.*, 2009).

570 Metabolism and immunity are thus affected when fish are under stress and this together
571 with sub-optimal dietary nitrogen formulations may affect fish welfare. A suitable
572 balanced diet should provide adequate health and immune conditions to fish. However,
573 little research has been done in optimizing the nitrogenous fraction of the diet in order
574 to minimize welfare problems. Recent evidence shows that stressful husbandry conditions
575 affect amino acid metabolism in fish, and under some stress situations an increase in the
576 requirement of certain indispensable amino acid may occur (Conceiçao *et al.*, 2012).
577 Protein or amino acid requirements may also increase as a direct consequence of
578 metabolic changes associated with inflammation and infection (Melchior *et al.*, 2004;
579 Sakkas *et al.*, 2013). In fact, the dependence of the immune system upon the availability
580 of amino acid is related to their role as signalling molecules essential for cellular
581 function (Li *et al.*, 2009). For instance, amino acids present important features in higher
582 vertebrates as they regulate i) activation of T- and B-lymphocytes, natural-killer cells and
583 macrophages; ii) cellular redox stage, gene expression and lymphocyte proliferation; and
584 iii) production of antibodies, cytokines and cytokines substances as NO and superoxide
585 (Wu, 2013). Moreover, both innate and adaptive immune responses are dependent upon
586 adequate provision of amino acids for the synthesis of antigen-presenting molecules,
587 immunoglobulins and cytokines (Sakkas *et al.*, 2013).

588 Tryptophan in particular is an essential amino acid with recognized roles in both
589 neuroendocrine and immune systems. Stress suppresses fish immunity and emerging

590 evidences (see above) show that dietary tryptophan is required to cope with stress
591 (Aragao *et al.*, 2008; Costas *et al.*, 2008); also, 5HT, melatonin and N-acetylserotonin,
592 products of tryptophan catabolism, can enhance host immunity by inhibiting the
593 production of superoxide, scavenging free radicals and attenuating the production of pro-
594 inflammatory cytokines (Perianayagam *et al.*, 2005). However, tryptophan nutrition should
595 be carefully considered since recent evidence showed opposite outcomes. Azeredo *et al.*
596 (2016) tested the effects of tryptophan on the innate immune response of Senegalese sole
597 (*Solea senegalensis*) in an attempt to explore the links between immune and
598 neuroendocrine responses and to evaluate possible beneficial effects on disease resistance.
599 It was observed that tryptophan supplementation (i.e. a level of 2x above tryptophan
600 requirements) may promote the immune status and disease resistance in fish reared at
601 low (12.5 kg/m^2) or high (31 kg/m^2) densities. However, an even higher increase in
602 tryptophan supplementation (i.e. a level of 4x above tryptophan requirements) showed to
603 be beneficial in chronically stressed fish, whereas undisturbed individuals showed a
604 compromised immune status and lower disease resistance. Likewise, Costas *et al.* (2013)
605 observed a positive effect of tryptophan supplementation (i.e. a 0.5% increase in a dry
606 matter basis), which in combination with arginine, threonine and lysine, mitigated the
607 immunosuppressive effects attributed to chronic stress action by counteracting the
608 decrease in plasma lysozyme, complement and peroxidase activities observed in stressed
609 fish fed a non-supplemented diet. Moreover, *L. rohita* juveniles decreased total circulating
610 leucocytes and serum lysozyme activity following exposure to a sub-lethal concentration
611 of nitrite-nitrogen for 45 days, which was counteracted by dietary tryptophan (i.e. 0.75
612 and 1.5% increase in a dry matter basis) surplus (Ciji *et al.*, 2015). Akhtar *et al.*
613 (2013a) and Akhtar *et al.* (2013b) showed that long-term (60 d) tryptophan
614 supplementation inhibits or suppresses chronic (15 d) thermal and/or salinity stress-
615 induced decrement in blood white blood cell count and respiratory burst activity; serum
616 total protein, albumin, globulin and lysozyme in *L. rohita*. Such effects might be due to
617 mitigation of stress response (serum cortisol) and suppression of oxidative stress (SOD
618 and CAT activity). In contrast, *Sander lucioperca* juveniles submitted to emersion stress
619 and long-term tryptophan treatment decreased both growth performance and plasma
620 lysozyme activity after 91 days of feeding (Mandiki *et al.*, 2016), highlighting again the

complexity of tryptophan effects in fish. All these studies suggest that immunosuppressive conditions increase dietary tryptophan requirement (Fig. 2).

Thus, dietary tryptophan appears to modulate fish immune responses depending on the species, level of supplementation in the diet and administration time when fish are reared under standard husbandry conditions (i.e. undisturbed fish). *S. senegalensis* fed a tryptophan supplemented diet (i.e. 0.5% increase in a dry matter basis) augmented plasma lysozyme levels after 14 days of feeding whereas no changes were observed following 28 days of tryptophan treatment (Costas *et al.*, 2012). Interestingly, *S. senegalensis* fed a similar diet with the same level of tryptophan supplementation increased plasma peroxidase activity after 18 days of feeding, with no changes in plasma lysozyme or complement activities. *D. labrax* fed a tryptophan supplemented diet (i.e. 0.5% increase in a dry matter basis) for 14 days were unable to improve both cellular (e.g. circulating leucocyte numbers) and humoral (e.g. nitric oxide levels) mediated immune responses (Machado *et al.*, 2015). Similarly, *A. persicus* fed a 0.5% increase of tryptophan (dry matter basis) did not change serum lysozyme and hemolytic activities after 5, 10 and 15 days of feeding (Hoseini *et al.*, 2016). In contrast, several dietary tryptophan levels induced detrimental responses in the young *C. idella* after 8 weeks of feeding (Wen *et al.*, 2014; Jiang *et al.*, 2015). For instance, tryptophan deficiency or surplus negatively affected gills and gut integrity by changing tight-junction proteins transcripts, which were probably related to an increased gene expression of pro-inflammatory cytokines and oxidative damage, compared to fish fed an optimum tryptophan level.

Tryptophan catabolism seems to play a critical role in the functions of both macrophages and lymphocytes during infection. In mammals, while the protein-related pool of tryptophan is kept constantly balanced between the processes of protein catabolism and protein synthesis, about the 99% of tryptophan dietary intake is routed to the kynurenine pathway (KP) of tryptophan catabolism (Macchiarulo *et al.*, 2009). IDO catalyses the first and rate-limiting step of the KP, and tryptophan catabolism by IDO seems to have a major immunomodulatory effect during inflammation in mammals (Le Floc'h *et al.*, 2011). In fact, tryptophan concentrations drop progressively in the plasma of animals with inflammation (Melchior *et al.*, 2004). The activation of IDO present in macrophages

is induced by cytokines released by activated immune cells and leads to tryptophan depletion. IDO activity is also responsible for superoxide anions consumption thus exerting direct antioxidant activity (Le Floc'h *et al.*, 2011). In fish, few studies have explored the role of tryptophan during inflammation and infection. Changes in the relative levels of metabolites such as choline, glycerophosphorylcholine and betaine, which are metabolites involved in tryptophan metabolism, were observed by ^{1}H nuclear magnetic resonance in the head-kidney of *S. salar* submitted to *Aeromonas salmonicida* infection (Liu *et al.*, 2016). *D. labrax* fed a 0.5% (dry matter basis) tryptophan surplus in the diet for 14 days and submitted to an intraperitoneal injection of *Photobacterium damselae* subsp. *piscicida* decreased cellular responses and dropped plasma nitric oxide and bactericidal activity compared to fish fed a control diet (Machado *et al.*, 2015). These authors hypothesized that a drop in nitric oxide levels could be related to the inhibitory effect of 3OH-anthranilic acid (a tryptophan metabolite) on the expression of inducible nitric oxide synthase, one of the main isoforms responsible for nitric oxide production (Chiarugi *et al.*, 2003). Machado *et al.* (2015) also observed a decreased lymphocyte recruitment to the inflammatory focus at 24 h after injection with the phlogistic agent in fish fed the tryptophan surplus compared to fish fed the control diet. It has been hypothesized that this drop in lymphocyte numbers could be linked to a decrease in T cells proliferation due to high expression of IDO in antigen presenting cells. This enzyme can be expressed not only by macrophages but also in dendritic cells (DCs), and IDO^{+} DCs in mammals can potently and dominantly be suppressive for T cell activation (Sharma *et al.*, 2007). Indeed, the tryptophan degrading enzyme IDO plays an important immunoregulatory role in higher vertebrates, and therefore in depth studies are required to unravel its role in fish immune responses.

Since both stress and tryptophan are known to modulate immune function, an immunomodulatory role for 5HT can be expected. In fact, this particular neurotransmitter has been shown to be involved in both mammalian and teleost immune function (Khan & Deschaux, 1997; Mössner & Lesch, 1998). For instance, scientific evidence showed that 5HT can regulate a variety of immune responses including up-regulation of mitogen-stimulated T- and B-lymphocyte proliferation (Aune *et al.*, 1994; Iken *et al.*, 1995; Young & Matthews, 1995). Moreover, 5HT also appears to act as an immunomodulator

682 for its ability to stimulate or inhibit inflammation (Cloëz-Tayarani *et al.*, 2003). However,
683 the functional role of 5HT in the fish immune system has been investigated much less.
684 Immunosuppressive effects induced by 5HT have been observed on the LPS- and
685 phytohaemagglutinin-stimulated proliferation of *O. mykiss* lymphocytes (Ferriere *et al.*,
686 1996). Those same authors also described that $5HT_{1A}$ receptors are expressed in trout
687 lymphocytes after mitogenic stimulation. Similarly, Duffy-Whritenour and Zelikoff (2008)
688 reported that endogenous (*in vivo*) and exogenous (*in vitro*) alterations in 5HT can
689 suppress splenic T- and B-lymphocyte proliferation in the *Lepomis macrochirus*, and
690 suggested a link between 5HT and the immune system in this particular teleost model. A
691 number of studies have also reported changes in the distribution of neuropeptides and
692 neurotransmitters in the digestive tract of parasitized fishes. For instance, Dezfuli *et al.*
693 (2000) and Dezfuli *et al.* (2008) observed 5HT immunoreactivities in immuno-related
694 cells of the intestine wall of parasitized *S. trutta*. Nevertheless, data proving evidence for
695 the role of 5HT in regulating the fish immune response is scarce and further studies
696 should focus on functional aspects such as cytokines secretion through the activation of
697 5HT receptors in fish leucocytes.

698 As commented above, melatonin is synthesized through 5HT and is involved in the
699 regulation of several important physiological and behavioural functions, including the
700 mammalian circadian rhythm, patterns of sleep, thermoregulation, reproduction and also
701 immune function (Carrillo-Vico *et al.*, 2005). Regarding its role in immune function,
702 melatonin is for instance involved in the development of thymus and spleen and also
703 modulates innate and adaptive immune responses (Carrillo-Vico *et al.*, 2005). Still,
704 knowledge regarding the actual fate of melatonin in the teleost immune functioning is
705 scarce. Esteban *et al.* (2006) reported clear effects of photoperiod on the humoral innate
706 immune system in two teleost fish, *Sparus aurata* and *D. labrax*, and provided evidence
707 that in fish, as in mammals, melatonin appears to play an important role in modulating
708 immune responses. Interestingly, seasonal changes in melatonin synthesis correlated with
709 changes in total white blood cell numbers and lysozyme activity in *O. mykiss*, but not
710 with the synthesis of ROS during the respiratory burst (Morgan *et al.*, 2008). *S. aurata*
711 intraperitoneally injected with melatonin (1 or 10 mg melatonin/kg of body weight)
712 increased the activity of peroxidase, phagocytosis and ROS production whereas the

713 expression of immune-relevant genes such as interleukin-1β, major histocompatibility
714 complex, virus-related response (interferon-regulatory factor-1 and Mx) and lymphocyte
715 markers (immunoglobulin M and T-cell receptor for B and T lymphocytes, respectively)
716 was up-regulated in the head–kidney of melatonin-injected fish 1 and 3 days post-
717 injection and decreased after 7 days (Cuesta *et al.*, 2008). *In vitro* studies showed that *S.*
718 *aurata* and *D. labrax* head-kidney leucocytes incubated with low (similar to
719 physiological) doses of melatonin did not change innate immune parameters, whereas
720 very high (pharmacological) doses inhibited peroxidase activity and increased ROS
721 production (Cuesta *et al.*, 2007). The melatonin receptors have been cloned in fish, and
722 partial or full-length sequences for the three high affinity melatonin receptor subtypes,
723 MT1, MT2 and Mel1c, have been obtained for several fish species (Esteban *et al.*,
724 2013). The presence of MT1 receptors on carp leukocytes as well as significant effects
725 of melatonin on leucocyte migration and apoptosis during zymosan-induced peritonitis
726 were also observed in *C. carpio* (Kepka *et al.*, 2015). Those authors described a potent
727 anti-inflammatory function for this hormone, whereas the observed melatonin induced
728 inhibition of leucocyte apoptosis indicates towards a dual function. Indeed, scientific
729 evidence suggests that neuroendocrine/immune interaction via melatonin is evolutionary
730 conserved but further studies are needed to clarify the role of melatonin in teleost
731 immune function.

732 **The kynurenine and niacin pathway**

733 As mentioned before, the KP is the dominant route for tryptophan degradation,
734 accounting for almost 95 % of the tryptophan metabolized. Furthermore, it is consider to
735 be the pathway through which excess tryptophan is removed from the organism, as the
736 highest enzymatic activities occur in the liver where the portal blood supply is readily
737 absorbed by the hepatocytes (Fernstrom, 2016).

738 Tryptophan is firstly oxidized by one of two possible enzymes (Fig. 1): TDO (also
739 known as pyrrolase) or IDO. The produced N-formylkynurenine is then converted to
740 kynurenine by arylformamidase, which can be metabolized by kynurenine
741 aminotransferase and thereby be converted into the bioactive kynurenic acid. However,
742 because this enzyme's K_m is very high, this branch of the KP will only occur in the

743 presence of tryptophan supplementation (Fernstrom, 2016). Instead, kynurenine can be
744 further transformed in 3-hydroxykynurenine and 3-hydrxyanthranilic acid (both are
745 biologically active compounds) which will finally be oxidized into acroleyl aminofumarate
746 (2-amino-3-carboxymuconate semialdehyde). The following metabolic steps highly rely on
747 the organism uptake of dietary niacin. Acroleyl aminofumarate can either be fully
748 oxidized to picolinic acid and acetyl-CoA or it can be spontaneously cyclized to
749 quinolinic acid and subsequently to nicotinamide and nicotinic acid (Fernstrom, 2016;
750 Grohmann *et al.*, 2003; Moffett & Namboodiri, 2003a). In fish, tryptophan conversion to
751 niacin is limited and deficiency is known to impair growth and antioxidant capacity in
752 *C. idella* (Li *et al.*, 2016a; Li *et al.*, 2016b). Also, supplementing *Ictalurus punctatus*,
753 diets devoid of niacin with graded levels of tryptophan did not enhance this vitamin
754 endogenous synthesis (Ng *et al.*, 1997). While these are well-established steps in the
755 mammalian KP, only some of the components have been identified so far in teleost fish
756 (Cortés *et al.*, 2016; Serrano & Nagayama, 1992; Yambe *et al.*, 2006; Yuasa *et al.*,
757 2015). Therefore, whether the exact sequence of events is present and which mechanisms
758 regulate enzyme activities in lower vertebrates, is a matter of further investigation.

759 The two leading enzymes (TDO or IDO) differ in tissue distribution, substrate affinity
760 and specificity as well as regulation. TDO, almost exclusive of the liver, is more widely
761 conserved than IDO gene sequences and has already been sequenced in a few fish
762 species [*S. salar, Oryzias latipes, Danio rerio* and *O. mykiss*; Serrano and Nagayama
763 (1992)]. It is highly specific for tryptophan but its affinity is lower compared to IDO's
764 (Le Floc'h *et al.*, 2011). Therefore, TDO reacts only to elevated hepatic amount of
765 tryptophan, particularly when it exceeds the requirement for protein and serotonin
766 synthesis (Murray, 2003). It is rate-limiting in this pathway and its activity is not only
767 induced by substrate availability, but also by glucocorticoids and glucagon (Badawy,
768 2002).

769 TDO activity is suppressed when IDO's is induced (Le Floc'h *et al.*, 2011). IDO's
770 affinity to tryptophan is higher than TDO's, but it is not as specific towards this amino
771 acid. In fact, it is also able to accept other indole substrates such as 5HT, 5-
772 hydroxytryptophan or tryptamine (Yuasa *et al.*, 2015). Although the contribution of IDO

to overall activity of KP is small when compared to that of TDO, it acquires great significance in conditions of immune stress. Indeed, tryptophan increasing levels do not enhance IDO activity per se. Instead, IDO is induced by inflammatory stimulus such as LPS or IFN-γ, which has been observed both in mammals (Grohmann *et al.*, 2003) and fish (Cortés *et al.*, 2016). A growing interest has been laid on the KP, particularly when initiated by IDO, due to important biological effects carried out by kynurenine metabolites on the immune response (Grohmann *et al.*, 2003; Moffett & Namboodiri, 2003b; Munn & Mellor, 2013) which makes this not only a tryptophan degradative pathway but an important biosynthetic pathway, too.

The gene expression of IDO is ubiquitous in the rainbow trout, with the lowest levels being detected in the spleen and kidney (Cortés *et al.*, 2016). Its distribution is also highly associated to its biological importance. Strong expression patterns in mucosal tissues of the *O. mykiss* (skin, gills and gut) (Cortés *et al.*, 2016) and its presence in brain and gut macrophages and dendritic cells (Le Floc'h *et al.*, 2011) reveal the importance of IDO-mediated tryptophan metabolism in immune tolerance-leading mechanisms indispensable in tissues where the host is in close and constant contact with an array of microorganisms. Besides depleting tryptophan from the microenvironment, which prevents microorganisms from utilizing it (Yuasa *et al.*, 2007), immune tolerance is carried out by tryptophan metabolites such as 3-hydroxykynurenine, 3-hydroxyanthranilic acid and quinolinic acid which, among other effects, are able to regulate T cell function and to modulate the oxidative status (Frumento *et al.*, 2001).

Interestingly, a completely different physiological role has been attributed to kynurenine which is that of being a pheromone. (Yambe *et al.* (2006)) observed that L-kynurenine present in the urine of female ovulating *Oncorhynchus masou* is responsible for attracting males, acting as a non-hormonal pheromone.

Conclusions and future perspectives

Tryptophan participates in different metabolic pathways involved in an array of physiological functions. Moreover, tryptophan can be directly transformed into other metabolites with important biological functions, including hormones such as melatonin or

neurotransmitters/neuromodulators as 5HT, contributing even more to the versatility of this compound. Even when the literature available on the effects and requirements of this amino acid is extensive, several aspects of tryptophan physiology remain to be elucidated. Recent research showed that, besides species-dependent differences, the tryptophan dietary requirements may also vary depending on other factors such as the stress suffered by the fish or the water quality, highlighting the need of a proper evaluation of those factors when assessing the dietary requirements of a given species. Available data somehow suggest that the teleosts' tryptophan requirement may increase under stressful conditions because of the beneficial effects of this amino acid to suppress stress and toxicant effects, and to improve antioxidant and immune status (Fig. 2). However, in many cases, mechanistic studies are still needed to know to which extent those effects are directly attributable to tryptophan or to related compounds such as 5HT, melatonin, among others. Furthermore, the effects of tryptophan on those physiological aspects have been shown to be very complex and to depend on species, dose and administration timing, and negative effects associated to tryptophan supplementation have been also reported. Therefore, even when tryptophan supplementation could be a promising strategy to improve fish conditions in aquaculture, specific studies to find proper doses and application time for the different farmed species are still needed to optimize its use as a welfare-promoting supplement.

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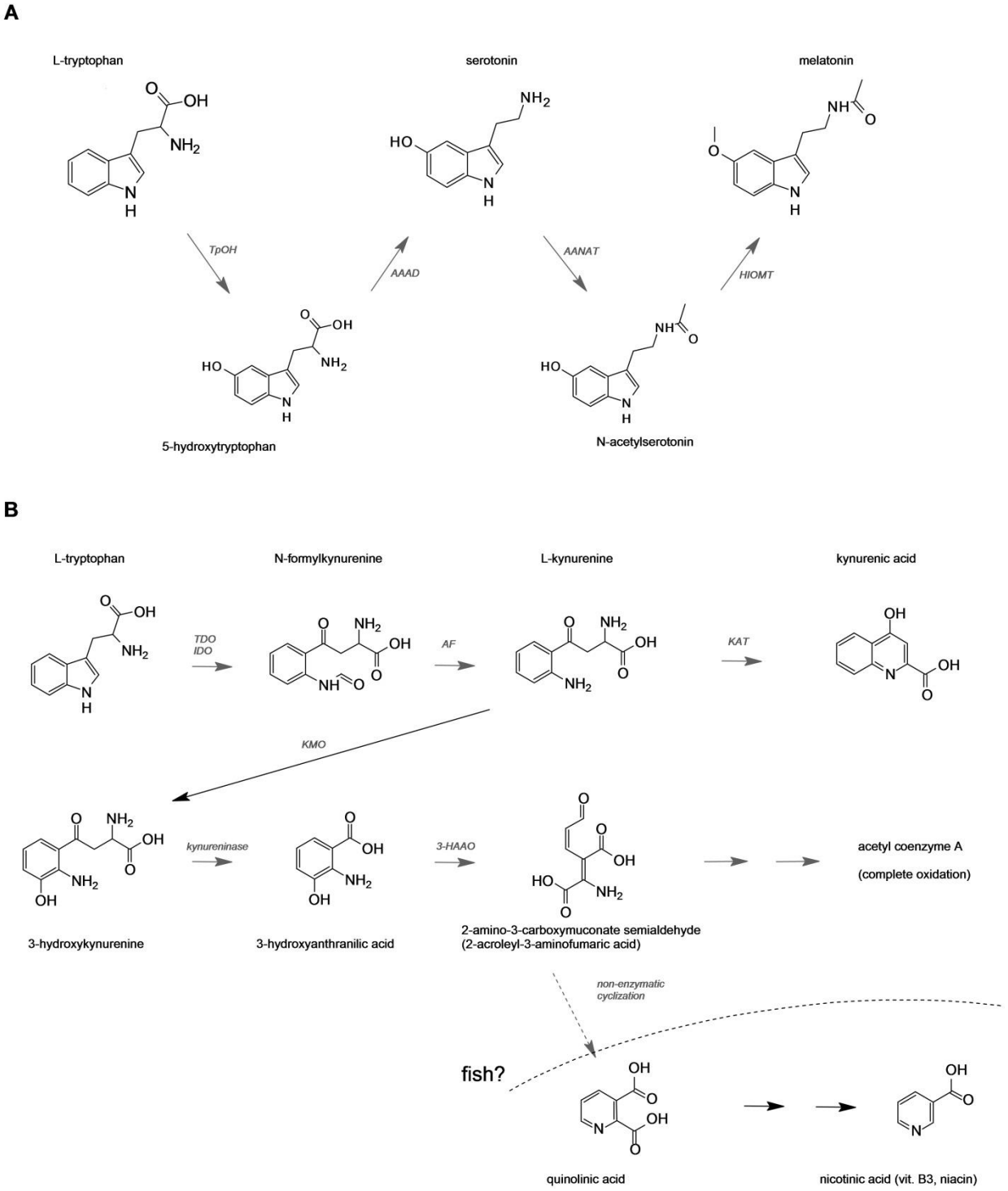
1471 Tables

1472 Table 1: Dietary tryptophan requirement (per cent of dietary protein level) in different
1473 teleosts

Species	Requirement	Protein source[1]	Reference
Catla catla	0.95	AA	(Ravi & Devaraj, 1991)
Ictalurus punctatus	0.50	CS, GL, AA	(Wilson *et al.*, 1978)
Clarias gariepinus	1.10	CS, GL, AA	(Fagbenro & Nwanna, 1999)
Heteropneustes fossilis	0.80	CS, GL, AA	(Ahmed, 2012)
Lates calcarifer	0.41	FM, SM, GL, AA	(Coloso *et al.*, 2004)
Oncorhynchus mykiss	0.45	FM, GL, AA	(Walton *et al.*, 1984)
	0.40	FM, GL	(Walton *et al.*, 1986)
	0.57-0.71	CS, GL, AA	(Kim *et al.*, 1987)
Oncorhynchus tshawytscha	0.50	UN	(Wilson, 2002)
Oncorhynchus kisutch	0.50	UN	(Wilson, 2002)
Oncorhynchus keta	0.70	CS, AA	(Akiyama *et al.*, 1985)
Oncorhynchus nerka	0.50	UN	(Wilson, 2002)
Cyprinus carpio	0.30	ZN, AA	(Dabrowski, 1981)
	1.10	CS, GL, AA	(Tang *et al.*, 2013)
Chanos chanos	0.60	FM, GL, AA	(Coloso *et al.*, 1992)
Morone chrysops× *M. saxatilis*	0.60-0.70	FM, AA	(Gaylord *et al.*, 2005)
Oreochromis niloticus	1.00	CS, GL, AA	(Santiago & Lovell, 1988)
	1.03	PD, AA	(Zaminhan *et al.*, 2017)
Labeo rohita	0.90-1.13	CS, GL, AA	(Fatma Abidi & Khan, 2010; Murthy & Varghese, 1997)
Cirrhinus mrigala	0.95-1.20	CS, GL, AA	(Ahmed & Khan, 2005; Benakappa & Varghese, 2003)
Ctenopharyngodon idella	1.27-1.30	FM, CS, GL, AA	(Wen *et al.*, 2014)
Sciaenops ocellatus	0.80	DMM, AA	(Pewitt *et al.*, 2016)

1474 [1] AA: amino acid mixture; CS: casein; GL: gelatine; FM: fishmeal; SM: squid meal;
1475 ZN: zein; PD: practical diet containing corn meal, soybean meal, poultry by-product,
1476 corn gluten; DMM: red drum muscle meal; UN: unknown.

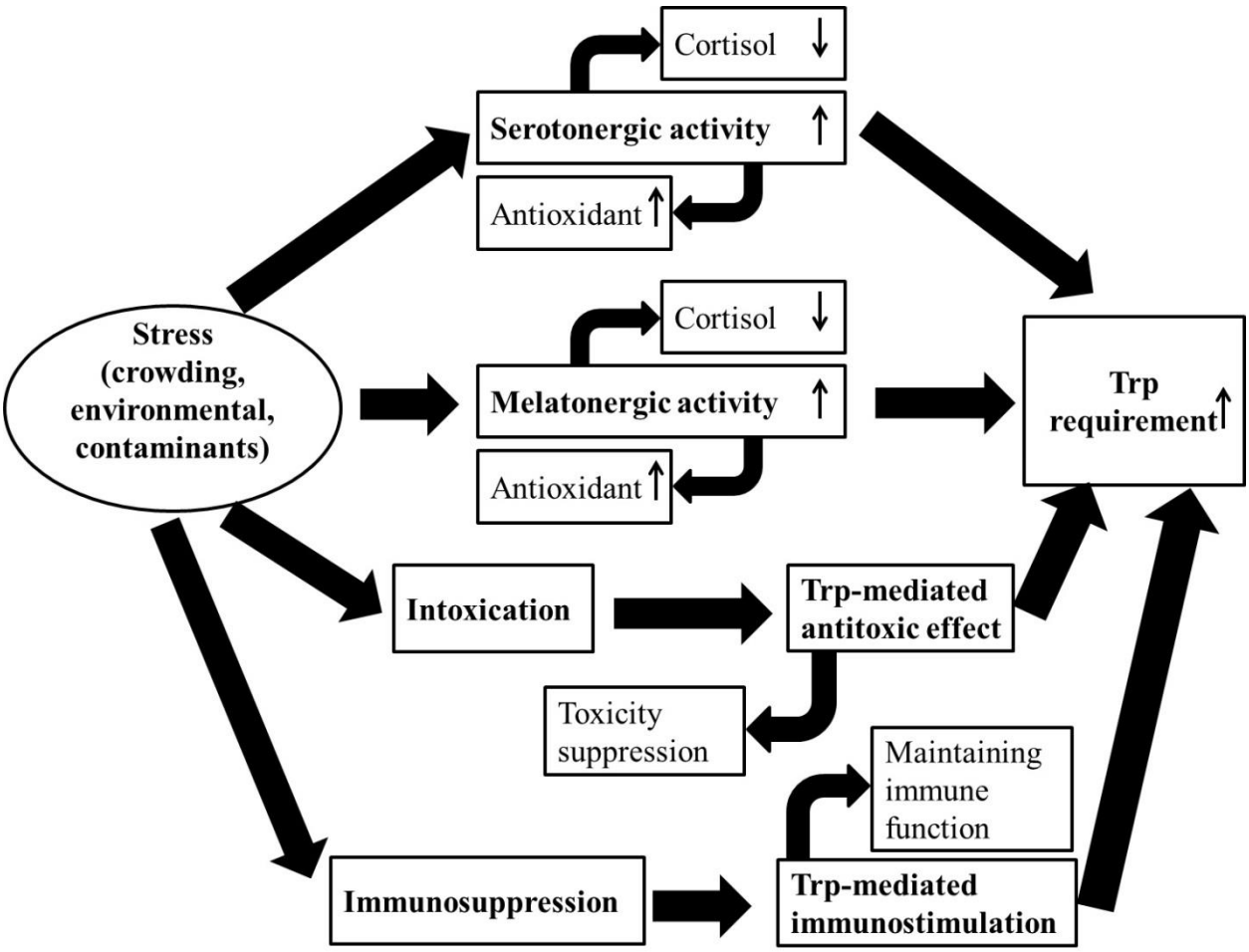
1477



1478

1479 Fig. 1. General L-tryptophan metabolic routes (besides its role as a component for
1480 protein synthesis) in vertebrates. A) Serotonin and melatonin biosynthetic pathways from

L-tryptophan and B) Main catabolic route for L-tryptophan (kynurenine pathway) (Badawy, 2017; Falcón *et al.*, 2010; Fernstrom, 2016; Macchiarulo *et al.*, 2009). In fish, the routes leading to the production of serotonin and melatonin are well established and known to be similar to those of other vertebrates. However, further research is still needed regarding the functionality of the kynurenin-niacin pathway, especially in what concerns the branch leading to the production of niacin (separated by a red line and arrow in the picture), which is well established in mammals but has not been confirmed in fish as yet (see the main text for further details). *TpOH*: tryptophan hydroxylase; *AAAD*: aromatic amino acid decarboxylase; *AANAT*: aryl-alkylamine-N-acetyltransferase; *HIOMT*: hydroxyindole-*O*-methyltransferase; *TDO*: tryptophan 2,3-dioxygenase; *IDO*: indoleamine 2,3-dioxygenase; *AF*: arylformamidase; *KAT*: kynurenine aminotransferase; *KMO*: kynurenine 3-monooxygenase; 3-*HAAO*: 3-hydroxyanthranilic acid 3,4-dioxygenase.



1494

1495 Fig. 2: Factors affecting teleosts' dietary tryptophan requirement

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